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A mammalian 2-5A system functions as an antiviral pathway in transgenic plants

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Abstract: Resistance to virus infections in higher vertebrates is mediated in part through catalysis of RNA decay by the interferon-regulated 2-5A system. A functional 2-5A system requires two enzymes, a 2-5A synthetase that produces 5'-phosphorylated, 2',5'-linked oligoadenylates (2-5A) in response to double-stranded RNA, and the 2-5A-dependent RNase L. We have coexpressed these human enzymes in transgenic tobacco plants by using a single plasmid containing the cDNAs for both human RNase L and a low molecular weight form of human 2-5A synthetase under control of different, constitutive promoters. Expression of the human cDNAs in the transgenic plants was demonstrated from Northern blots, by specific enzyme assays, and by immuno-detection (for RNase L) of leaves, detached or in planta, of the coexpressing transgenic plants by tobacco mosaic virus, alfalfa mosaic virus, or tobacco etch virus resulted in necrotic lesions. In contrast, leaves expressing 2-5A synthetase or RNase L alone and leaves containing the plasmid vector alone produced typical systemic infections. While alfalfa mosaic virus produced lesions only in the inoculated leaves regardless of the concentration of virus in the inoculum, high, but not low, levels of tobacco etch virus inoculum resulted in escape of virus to uninoculated leaves. Nevertheless, there was a substantial reduction of tobacco etch virus yield as measured by ELISA assay in the coexpressing transgenic plants. These results indicate that expression of a mammalian 2-5A system in plants provides resistance to virus infections.

The interferon family of cytokines induces an antiviral state in cells of higher vertebrates (1). Virus-infected cells produce and secrete interferons α and β, which signal uninfected cells that a virus infection has occurred. Interferons bind to specific cell surface receptors, activating Jak-STAT signal transduction pathways that induce interferon-stimulated genes (2). The proteins encoded by these genes are responsible for the biological effects of interferons, including the antiviral responses. The 2-5A system (3) is one such interferon-induced antiviral pathway present in cells of reptiles, avians, and mammals (4). Two types of enzymes are essential for a functional 2-5A system: (i) any one of several 2-5A synthetases, which require double-stranded (dsRNA) to produce 2-5A, a series of 5'-phosphorylated, 2',5'-linked oligoadenylates (5, 6) and (ii) the 2-5A-dependent RNase L (7-9). 2-5A activates RNase L which cleaves viral and cellular single-stranded RNAs, predominantly after UpUp sequences (10, 11). Because virus-infected cells often contain dsRNA activators of 2-5A synthetase (12), for instance as replicative intermediates of RNA viruses, RNA degradation by RNase L frequently occurs in interferon-treated virus-infected cells (13, 14). It is well established that the 2-5A system inhibits replication of the picornaviruses, encephalomyocarditis virus, and mengo virus in interferon-treated mammalian cells (15-18).

Plants appear to lack an equivalent of the mammalian 2-5A system. Kerr and colleagues (4) could not detect RNase L, assayed by 2-5A binding activity, in tobacco, Nicotiana glutinosa and Nicotiana tabacum. Furthermore, in the same study, no 2-5A synthetase was detected nor was any 2-5A detected in control, tobacco mosaic virus (TMV)-infected, interferon-treated or poly(I)-poly(C)-treated plants. The absence of a 2-5A system in control tobacco plants is verified in this study (Figs. 2-5). Previously, expression in potato of rat 2-5A synthetase mRNA, without expression of RNase L, was correlated with partial resistance to potato virus X in some but not all transgenic plants (19). The biochemical basis for both this effect and for an anti-TMV effect of 2',5'-oligoadenylate in nontransgenic N. glutinosa leaf disks was not established (20). Although plant oligoadenylates do exist, they differ substantially from 2-5A (21). Accordingly, the present work was undertaken to genetically engineer virus-resistant plants by expression of a mammalian 2-5A system consisting of both 2-5A synthetase and RNase L.

Materials and Methods

Construction of the Expression Vector and Transformation of Plants. The coding regions for a low molecular weight (40 kDa) species of human 2-5A synthetase and for human RNase L were obtained from plasmids pBabe.EI.6S (a gift from B. R. G. Williams, The Cleveland Clinic Foundation) and pZC5 (9), respectively. Coexpression of these cDNAs was engineered by subcloning into the binary (22) plant transformation/expression vector, pAM2200. This construct contained a 1321 bp EcoRI fragment of 2-5A synthetase cDNA and a 2249-bp HindIII fragment of RNase L cDNA, each containing the complete coding sequences. Both the fragments were modified by PCR to contain a GCCACC sequence immediately preceding to the ATG translation start site to provide a better context for plant cell translation. In addition, 2-5A synthetase contained an additional GGG codon after the ATG codon. The inserted fragments were partially sequenced across the junctions to confirm proper fusion of the fragments. The binary plasmid also contained a neomycin phosphotransferase plant selectable marker gene driven by the transferred DNA (T-DNA) gene nopaline synthase promoter (23). This plasmid was used to produce transgenic tobacco plants by Agrobacterium-mediated transformation of axenically grown tobacco seedlings (24). Transformed tobacco plants were selected on Murasighe & Skoog (MS) (25) plates containing 300 μg of kanamycin per ml.

Abbreviations: dsRNA, double-stranded RNA; TMV, tobacco mosaic virus; TEV, tobacco etch virus; AIMV, alfalfa mosaic virus.

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Southern and Northern Blots of Plants Detect Integration and Expression of the Transgenes. Integration of the plasmid DNA into plant DNA was determined by Southern blot analysis (26). Five grams of fresh plant tissue was used to prepare DNA as described by Guillemaut and Maréchal-Drouard (27). Briefly, plant tissue was ground in liquid nitrogen and thawed in extraction buffer (100 mM sodium acetate, pH 4.8/50 mM EDTA, pH 8.0/500 mM NaCl/2% polyvinylpyrrolidone-40; the mixture adjusted to pH 5.5, then SDS was added to 1.4% and cysteine to 10 mM), incubated at 65°C for 20 min and centrifuged. One-half volume of 3 M potassium acetate (pH 4.8) was added to the supernatant, and the mixture was incubated at 0°C for 1 h. The DNA was recovered by ethanol precipitation, dissolved in TE buffer, and centrifuged overnight in a cesium chloride gradient. DNA was digested with HindIII, separated on a 1% agarose gel, and transferred to a Zeta-probe membrane (Bio-Rad) according to the manufacturer’s recommendations. The membrane was hybridized with a randomly labeled DNA probe (28) by using the human 2-5A synthetase and RNase L cDNAs as templates.

To determine expression of 2-5A synthetase and RNase L cDNAs in transgenic tobacco plants, total RNA was prepared from 15 independently transformed plants as described by Logemann et al. (29). RNA extraction buffer contained 8 M guanidinium chloride, 20 mM Mes, 20 mM EDTA, and 50 mM 2-mercaptoethanol (pH 7.0). Aliquots of RNA samples were separated on formaldehyde gels, transferred to the Zeta-probe membranes and probed with the same radiolabeled DNA fragments as in the Southern blots.

Detection of RNase L Protein by Western Blot. A total cellular extract was prepared by grinding transformed plant tissue in liquid nitrogen and thawing in protein extraction buffer (0.5 M sucrose/0.1% ascorbic acid/0.1% cysteine-HCl/0.01 M Tris-HCl, pH 7.5) (23) in the presence of 15 μg phenylmethylsulfonl fluoride. The homogenate was centrifuged at 14,000 × g for 30 min at 4°C, and the supernatant containing the soluble proteins was recovered and concentrated using a Microcon (Amicon) concentrator. Extracts (200 μg of protein) were separated on a 12.5% acrylamide gel (30) along with 200 ng recombinant RNase L protein as a standard. The resolved proteins were transferred to a nitrocellulose membrane using a Bio-Rad trans-blot apparatus at 250 mA for 1 h. The membrane was immersed in a blocking solution (5% nonfat dry milk in Tris-buffered saline) and incubated at 28°C for 1 h. The membrane was then washed and incubated with a 1:5000 dilution of a mouse monoclonal antibody prepared against human RNase L antigen (49). Following incubation with the antibody, the membrane was placed in the biotinylated goat anti-mouse antibody solution for 1 h at 28°C with gentle agitation. The membrane was washed and incubated in a streptavidin-biotinylated alkaline phosphatase complex for 1 h followed by immersion in a color development solution until purple bands became visible.

Determination of RNase L Activity in Intact Leaves. Leaves from plants expressing both or one of the cDNAs and control leaves were injected with 2-5A [2.5 μM pA(2′p5′)A5] in 12.5 μM phosphate buffer, pH 6.8] by using a fine needle (22 or 26 gauge; Becton Dickinson) attached to a tuberculin syringe. The injection was carried out by inserting the needle into leaf tissue close to a vein and injecting the solution until a part of lamina appeared water-soaked. This process was repeated several times within the entire leaf with infiltration in the solution. Presence of the solution in the intercellular spaces of leaf tissue could be readily detected by the water-soaked appearance of the leaf tissue. Because of leakage at the injection sites, it was not possible to determine exact amount of the solution that was retained in each leaf. However, the entire leaf was saturated with the solution by filling up intercellular spaces. Leaves were harvested 3 h after the injections and were frozen immediately in liquid nitrogen.

Total RNA was prepared from the frozen leaves as described above, fractionated on a formaldehyde-agarose gel, and transferred to a membrane. The RNA in the blots was hybridized to a 32P-labeled human 18S rRNA cDNA probe (18) (a gift of Bret A. Hassel, Baltimore).

Assay of 2-5A Synthetase Activity in Extracts of Leaves. Plant extracts were prepared by homogenizing leaves in Nonidet P-40 lysis buffer [0.5% (vol/vol) Nonidet P-40/50 mM KCl/5 mM magnesium acetate/20 mM Tris-HCl, pH 7.5/5 mM 2-mercaptoethanol/10 μg of leupeptin per ml]. Cell lysates were centrifuged at 10,000 × g for 10 min at 4°C, the supernatants were collected, and the protein concentration was determined as described (32). Extracts of interferon-treated (200 units per ml human interferon α for 18 h) human HeLa cells were prepared by the same method, except cells were disrupted by vortex mixing. 2-5A synthetase in the cell and leaf extracts were adsorbed to the activating affinity matrix, poly(1)-poly(C) cellulose as described (33, 34). Poly(1)-poly(C) covalently linked to cellulose was washed three times in buffer A [20 mM Tris-HCl, pH 7.5/50 mM KCl/5 mM magnesium acetate/5 mM 2-mercaptoethanol/10% (vol/vol) glycerol]. Protein extract from the leaves or HeLa cells, 200 μg per assay, was incubated with 11 μg poly(1)-poly(C) linked to cellulose in a final volume of 60 μl for 1 h on ice. After washing three times in buffer A by vortex mixing, centrifugation, and resuspension, the pellet was suspended in buffer A supplemented with 8 mM ATP and 16 mM magnesium chloride and incubated at 30°C for 24 h. After centrifugation, the supernatant was removed and assayed for 2-5A.

To measure 2-5A, the supernatants (5 μl each) or known amounts of authentic pA(2′p5′)A5 were incubated in a final volume of 25 μl with insect extract containing human, recombinant RNase L (31) (1 μg per assay) in the presence of 0.2 mM poly(U) (labeled at the 3′ termini with 32P)pCp (3000 Ci/mmol; 1 Ci = 37 GBq) and T4 RNA ligase as described (35). Reactions were performed at 30°C for 30 min in 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 8 mM 2-mercaptoethanol, 90 mM KCl, and 10 μg of leupeptin per ml. Reactions were terminated with 5 μl stop buffer, boiled for 5 min, centrifuged briefly, and 5 μl was loaded to 6% polyacrylamide/8 M urea gels. After electrophoresis, x-ray film was exposed to the gel.

Virus Inoculation of Plants. Transgenic plants were grown in an access-controlled greenhouse. R1 transgenic plants were examined for virus resistance by using both cut-leaf and in planta inoculation assays. Leaves from these plants were cut at the petiole and placed in Petri dishes on moist filter papers. Leaves maintained in Petri dishes for the incubation period remained in good physiological condition. The leaves were inoculated with a 1:100, 1:1000, and 1:2500 dilution of freshly prepared extracts from tobacco leaves systemically infected with tobacco etch virus (TEV, severe strain; ATCC PV69), TMV (common strain), and alfalfa mosaic virus (AIMV, Nebraska strain) respectively. Six of six control plants became systemically infected when inoculated with sap diluted 1:100, 1:1000, and 1:15000, respectively. Following inoculation with virus, the leaves were rinsed with water, and Petri dishes were sealed with stretched parafilm and placed in an incubator at 22°C for 7 days under 18-h light per day. In planta inoculation of greenhouse grown transgenic plants were carried out by using various dilutions of TEV and AIMV. A total of 33 and 29 kanamycin resistant transgenic plants were inoculated with TEV and AIMV, respectively. A single leaf was inoculated in each plant by using either TEV or AIMV. Virus titers in the TEV-inoculated and in upper uninoculated leaves were determined by ELISA by using TEV-specific polyclonal antibodies and purified TEV virions to construct standard curves (36). TEV antibodies were prepared in rabbit by using TEV virions as antigen.
RESULTS

Construction of Transgenic Plants Expressing Human RNase L and 2-5A Synthetase. To express a human 2-5A system in plants, a binary plant transformation-expression vector containing both the 2-5A synthetase and RNase L cDNAs under control of the cauliflower mosaic virus 35S promoter (37) and the chlorella virus adenine methyltransferase gene promoter (38), respectively, was constructed (Fig. 1). Young tobacco seedlings (N. tabacum, petit Havana SR-1) (39) were transformed by using the Agrobacterium cocultivation method (24). Cloning of the human 2-5A system cDNAs in these plants had no effect on the transformation frequency, growth, or development of the plants. All of the primary transformants (R0) that were grown to maturity and allowed to self-pollinate produced viable seeds. Kanamycin-resistant R1 seedlings were grown in a greenhouse and analyzed for transgene expression and virus resistance. Southern blot analysis of the transgenic plants showed that most of the kanamycin-resistant plants contained intact and unmodified inserts (Fig. 2A), indicating appropriate integration of the transgenes into plant chromosomes. However, one plant was observed to contain only the 2-5A synthetase cDNA and another plant contained only the RNase L cDNA (Fig. 2A, lanes 6 and 7, respectively). Northern blot analysis indicated that both the 2-5A synthetase and RNase L transgenes were transcribed in the transformed plants (Fig. 2B). RNA from the control tobacco plants containing the empty plasmid vector did not hybridize to either the 2-5A synthetase or RNase L radiolabeled cDNA probes (Fig. 2B, lane 1).

Production of Functional RNase L in the Transgenic Plants. The expression of RNase L in transgenic tobacco was monitored by probing Western blots with a monoclonal antibody to human RNase L (49). All transgenic plants expressing RNase L mRNA also expressed the nuclease protein (Fig. 3). RNase L comigrating with authentic human, recombinant RNase L (lane RL) was observed in plants containing both the synthetase and RNase L cDNAs (lanes 3-5) as well as in plants containing only the RNase L cDNA (lane 2) but not in plants containing the empty plasmid vector (lane C). The immunoblot showed a single protein band of about 80 kDa in protein extracts from transgenic plants.

To determine if the RNase L in the transgenic tobacco plants was functional, the integrity of the 18S rRNA was analyzed after injecting detached leaves with 2-5A (Fig. 4). Transgenic leaves were injected with 2.5 μM p2A(2'5'p5'2') and then incubated for 3 h before total RNA was prepared. Degradation of rRNA was demonstrated by probing the total RNA on a Northern blot with a radiolabeled cDNA to 18S rRNA. Injection of 2-5A resulted in enhanced degradation of rRNA in transgenic plants expressing both the 2-5A synthetase and RNase L (lane 6) or only RNase L (lane 5), but not in control plants transformed with vector alone (lane 1) or in plants expressing only 2-5A synthetase (lane 3). In addition, specific 2-5A binding activity was detected in transgenic plants expressing RNase L but not in control plants (data not shown). These data indicate that introduction of the human RNase L cDNA into transgenic plants led to production of a functional RNase L enzyme. Because of the requirement for dsRNA to activate 2-5A synthetase, coexpression of RNase L and 2-5A

![Fig. 1](image1.png)

![Fig. 2](image2.png)
monoclonal antibody markers in plants; 1, plasmid by itself, synthetase, both 2-5A synthetase protein. 

Expression of Functional 2-5A Synthetase in the Transgenic Plants. To determine if 2-5A synthetase was present in the transgenic tobacco plants, we performed functional assays for this enzyme. A lack of a high affinity antibody for human 40-kDa 2-5A synthetase precluded Western blot assays for this protein. 2-5A synthetase in extracts of leaves or in interferon-treated human HeLa cells was immobilized and activated by using the affinity matrix, poly(I)-poly(C)-cellulose (see Materials and Methods) (33, 34). Production of 2-5A oligonucleotides from ATP was then determined by the ability to activate recombinant human RNase L (31) (Fig. 5). For comparison, authentic 2-5A [pA(2'p5'A)3] was incubated with RNase L, resulting in the production of discrete poly(U) cleavage products (lanes 1–6). Partial cleavage of poly(U) was induced with 0.3 nM 2-5A, with maximal breakdown of poly(U) occurring at a concentration of 7 nM 2-5A. Control plants lacked detectable 2-5A synthetase activity, in accord with a previous report (lanes 7 and 8) (4). In contrast, 2-5A synthetase was clearly obtained in plants containing human 2-5A synthetase cDNA (lanes 9 and 10 are from a plant transgenic for both human 2-5A synthetase and RNase L). Identical poly(U) cleavage products were observed with authentic 2-5A (lanes 1–6), the transgenic plants (lane 9), and with 2-5A synthetase assays from interferon-treated human HeLa cells (lanes 11 and 12). In every instance, the cleavage of poly(U) required the addition of RNase L, thus ruling out the possibility that the breakdown of the RNA was due to contaminating ribonucleases. These results demonstrated production of nanomolar levels of functional 2-5A synthetase in transgenic assays performed on extract of the transgenic tobacco plants. Although these amounts of 2-5A are very low, they were more than sufficient to activate RNase L (Fig. 5, lane 9).

Coexpression of Human 2-5A Synthetase and RNase L Produces Antiviral Effects in Intact Tobacco Plants. To monitor viral resistance, leaves were manually inoculated with three different types of plant viruses: TEV, a member of the potyvirus group; TMV, a member of the tobamovirus group; and ALMV, a member of the bromovirus group (40). Leaves from a total of 48 kanamycin-resistant R1 progeny plants expressing both 2-5A synthetase and RNase L, or either proteins alone, were tested. A composite picture from representative plants is shown (Fig. 6). Infection of plants expressing both proteins induced necrotic local lesions (Fig. 6a, c, and e), in contrast plants expressing only 2-5A synthetase (Fig. 6g), only RNase L (Fig. 6h) and control plants containing the empty plasmid vector (Fig. 6b, d, and f) produced typical systemic infections. In nature, TEV and ALMV are not known to produce local lesions in tobacco plants (40). TMV does
but sometimes both upper and lower parts of the plants. Therefore, TEV virions escaped into vascular tissues from inoculated tissue when a high concentration inoculum was used. In planta infection by TMV had similar effects as TEV. Despite the escape of TEV from inoculated leaves, there was nevertheless a substantial antiviral effect in the transgenic plants. Quantitation of TEV production by an ELISA showed a significant antiviral effect in the R2 generation of coexpressing transgenic plants (Table 1). There was a 5- to 15-fold reduction of TEV production in the inoculated leaves and 16- to 35-fold reduction in the uninoculated upper leaves in comparison to infected control plants containing the empty plasmid vector. Data indicated the presence of virus only in the necrotic tissue but not in other areas (Table 1). However, at low levels of TEV inoculum, there was a 16- to 23-fold reduction in virus production in the inoculated leaves with no escape to upper leaves. In contrast to results obtained with TEV, no systemic symptoms were found in transgenic plants infected with AIMV, even 40 days after inoculation. In addition, AIMV infection of the coexpressing transgenic plants produced only local lesions and only on the inoculated leaves without veinal necrosis, even when high levels of inoculum (>500 lesions/leaf) was used (data not shown). Therefore, a substantial antiviral effect was retained in the R2 generation of coexpressing transgenic plants.

**DISCUSSION**

Results presented here provide a clear, visual demonstration that the expression of a mammalian 2-5A system restricts viral replication in transgenic plants. Although we show that coexpression of the two proteins, 2-5A synthetase and RNase L, is required for the formation of local lesions, the precise mechanism of action for the antiviral effect in plants remains to be elucidated. The most likely interpretation of these data is that viral dsRNAs activated the 2-5A synthetase resulting in RNA decay by RNase L thus limiting viral replication. Apparently, the very low levels of expression of these proteins, compared

<table>
<thead>
<tr>
<th>Plants</th>
<th>Inoculated leaves</th>
<th>Uninoculated upper leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Inoculum (1:100 dilution)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8.62 0.35</td>
<td>2.22 0.38</td>
</tr>
<tr>
<td>2</td>
<td>3.37 0.45</td>
<td>3.81 0.44</td>
</tr>
<tr>
<td>3</td>
<td>9.51 0.22</td>
<td>1.72 0.28</td>
</tr>
<tr>
<td>4</td>
<td>4.12 0.31</td>
<td>2.86 0.37</td>
</tr>
<tr>
<td>5</td>
<td>7.13 0.33</td>
<td>3.53 0.35</td>
</tr>
<tr>
<td>C</td>
<td>52.24</td>
<td>61.11</td>
</tr>
<tr>
<td>Low Inoculum (1:2000 dilution)</td>
<td>1</td>
<td>2.55 0.31</td>
</tr>
<tr>
<td>2</td>
<td>3.26 0.38</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.71 0.22</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.32 0.28</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.78 0.31</td>
<td></td>
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<tr>
<td>C</td>
<td>53.38</td>
<td>52.57</td>
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Plants 1–5, independently transformed kanamycin-resistant transgenic plants; C, control plants containing empty plasmid vector.

*Virus concentrations were determined 21 days after inoculation by ELISA without background correction by using standard curves constructed from purified viruses. A minimum of four leaves was assayed from each transgenic plants, and each sample was run in duplicate.

†Necrotic tissue contained both lesion and nonlesion areas.

‡No necrotic tissue.
for instance to interferon-treated, human cells, was sufficient to produce an antiviral response. Activation of the 2-5A system in plants could conceivably be achieved either by extensive secondary structure in viral RNAs or by double-stranded replicative intermediates formed during viral replication. With low, but not high, inoculae of TEV, and with both low and high inoculae of AIMV, there were lesions only on initially infected leaves. However, at high inoculae of TEV, virus escape occurred into the vascular system with translocation of virus to other parts of the plants. In either case, there was destruction of cells in vascular bundles and other tissues. Expression of the 2-5A system substantially reduced viral growth, even when plants were inoculated with high amounts of TEV (Table 1). We therefore conclude that the viruses used in this study caused the destruction of the infected cells in the coexpressing transgenic plants, leading to the formation of necrotic lesions and the observed antiviral effects.

Expression of the 2-5A system in plants has several apparent advantages compared to alternative plant antiviral strategies currently in use. The most common approach involves pathogen-derived resistance (42) in which plants are transformed to express certain viral-coded proteins or antisense RNAs (43, 44). A potential limitation of this approach has been virus recombination that may occur in transgenic plants and might yield a more virulent virus, expansion of the host range of a virus or vector specificity (45–47). These risks, in general, are not considered to be high (48). However, even in instances of substantial resistance to virus infection, protection is usually limited to the virus from which the protein or antisense RNA was derived, or to closely related strains. In contrast, the 2-5A system is predicted to provide resistance to any plant virus that produces dsRNA in the infected cell. Most plant viruses, including those in the economically important potyvirus group, are positive sense, single-stranded RNA viruses which produce a dsRNA intermediate during their replicative process. Furthermore, because plant viruses are na"ive to the 2-5A system, they are likely to be even more vulnerable to it than viruses of higher vertebrates.

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